

Immuno Inhibitory Pyrazolone Compounds

The present invention relates to pyrazolone compounds, to methods for their preparation, to compositions containing them, and to methods and use for 5 clinical treatment of medical conditions which may benefit from immunomodulation, e.g. rheumatoid arthritis, multiple sclerosis, diabetes, asthma, transplantation, systemic lupus erythematosus and psoriasis. More particularly the present invention relates to pyrazolone compounds, which are CD80 antagonists capable of inhibiting the interactions between CD80 and 10 CD28.

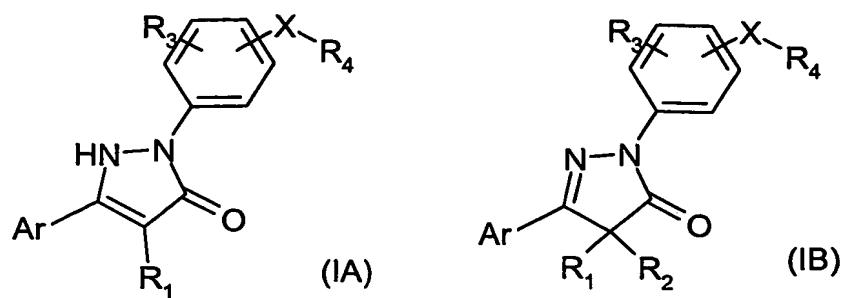
Background to the Invention

The immune system possesses the ability to control the homeostasis between the activation and inactivation of lymphocytes through various regulatory 15 mechanisms during and after an immune response. Among these are mechanisms that specifically inhibit and/or turn off an immune response. Thus, when an antigen is presented by MHC molecules to the T-cell receptor, the T-cells become properly activated only in the presence of additional co-stimulatory signals. In the absence of these accessory signals there is no 20 lymphocyte activation and either a state of functional inactivation termed anergy or tolerance is induced, or the T-cell is specifically deleted by apoptosis.

One such co-stimulatory signal involves interaction of CD80 on specialised 25 antigen-presenting cells with CD28 on T-cells, and this signal has been demonstrated to be essential for full T-cell activation. (Lenschow *et al.* (1996) *Annu. Rev. Immunol.*, **14**, 233-258). It would therefore be desirable to provide compounds which inhibit this CD80/CD28 interaction.

30 Detailed Description of the Invention

According to the present invention there is provided a compound of formula (IA) or (IB) or a pharmaceutically or veterinarily acceptable salt, hydrate or solvate thereof:



wherein

Ar represents an optionally substituted monocyclic or bicyclic aromatic or

5 heteroaromatic group having from 5 to 10 ring atoms,

R₁ and R₂ independently represent H, or C₁-C₆ alkyl;

R_3 represents H; F; Cl; Br; $-NO_2$; $-CN$; C_1-C_6 alkyl optionally substituted by F

10 or Cl; or C₁-C₆ alkoxy optionally substituted by F;

R₄ represents a carboxylic acid group (-COOH) or an ester thereof, or -C(=O)NR₆R₇, -NR₇C(=O)R₆, -NR₇C(=O)OR₆, -NHC(=O)NR₇R₆ or -NHC(=S)NR₇R₆ wherein

15 R_6 represents H, or a radical of formula $-(Alk)_m-Q$ wherein

m is 0 or 1

Alk is an optionally substituted divalent straight or branched C₁-

20 C₁₂ alkylene, or C₂-C₁₂ alkenylene, or C₂-C₁₂ alkynylene radical or a divalent C₃-C₁₂ carbocyclic radical, any of which radicals may be interrupted by one or more -O-, -S- or -N(R₈)- radicals wherein R₈ represents H or C₁-C₄ alkyl, C₃-C₄ alkenyl, C₃-C₄ alkynyl, or C₃-C₆ cycloalkyl, and

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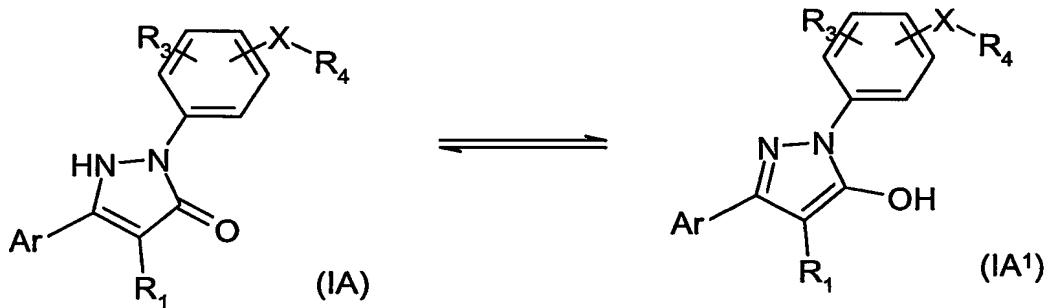
Q represents H; -CF₃; -OH; -SH; -NR₈R₈ wherein each R₈ may be the same or different, or form a ring when taken together with the nitrogen to which they are attached; an ester group; or an

optionally substituted aryl, aryloxy, cycloalkyl, cycloalkenyl or heterocyclic group; and

5 R₇ represents H or C₁-C₆ alkyl; or when taken together with the atom or atoms to which they are attached R₆ and R₇ form a monocyclic heterocyclic ring having 5, 6 or 7 ring atoms; and

10 X represents a bond or a divalent radical of formula -(Z)_n-(Alk)- or -(Alk)-(Z)_n- wherein Z represents -O-, -S- or -NH-, Alk is as defined in relation to R₆ and n is 0 or 1.

Compounds (IA) may exist in the form of tautomers (IA¹):



15 Hereafter, the compounds (IA) of the invention may be represented and referred to in either tautomeric form and it is to be understood that any and all tautomeric forms of structures (IA) and (IB), in particular (IA¹), are included in the invention.

20 Compounds of general formula (IA) and (IB) are CD80 antagonists. They inhibit the interaction between CD80 and CD28 and thus the activation of T cells, thereby modulating the immune response.

Accordingly the invention also includes:

25 (i) a compound of formula (IA) or (IB) or a pharmaceutically or veterinarily acceptable salt thereof for use in the treatment of conditions which benefit from immunomodulation.

(ii) the use of a compound of formula (IA) or (IB) or a pharmaceutically or
veterinarily acceptable salt thereof in the manufacture of a medicament for the
treatment of conditions which benefit from immunomodulation.,.

5 (iii) a method of immunomodulation in mammals, including humans,
comprising administration to a mammal in need of such treatment an
immunomodulatory effective dose of a compound of formula (IA) or (IB) or a
pharmaceutically or veterinarily acceptable salt thereof.

10 (iv) a pharmaceutical or veterinary composition comprising a compound of
formula (IA) or (IB) or a pharmaceutically or veterinarily acceptable salt
thereof together with a pharmaceutically or veterinarily acceptable excipient or
carrier.

15 **Conditions which benefit from immunomodulation include:**
Acute disseminated encephalomyelitis
Adrenal insufficiency
Allergic angiitis and granulomatosis
Amyloidosis

20 Ankylosing spondylitis
Asthma
Autoimmune Addison's disease
Autoimmune alopecia
Autoimmune chronic active hepatitis

25 Autoimmune haemolytic anaemia
Autoimmune Neutrogena
Autoimmune thrombocytopenic purpura
Behçet's disease
Cerebellar degeneration

30 Chronic active hepatitis
Chronic inflammatory demyelinating polyradiculoneuropathy
Chronic neuropathy with monoclonal gammopathy
Classic polyarteritis nodosa
Congenital adrenal hyperplasia

- Cryopathies
- Dermatitis herpetiformis
- Diabetes
- Eaton-Lambert myasthenic syndrome
- 5 Encephalomyelitis
- Epidermolysis bullosa acquisita
- Erythema nodosa
- Gluten-sensitive enteropathy
- Goodpasture's syndrome
- 10 Guillain-Barre syndrome
- Hashimoto's thyroiditis
- Hyperthyroidism
- Idiopathic hemachromatosis
- Idiopathic membranous glomerulonephritis
- 15 Isolated vasculitis of the central nervous system
- Kawasaki's disease
- Minimal change renal disease
- Miscellaneous vasculitides
- Mixed connective tissue disease
- 20 Multifocal motor neuropathy with conduction block
- Multiple sclerosis
- Myasthenia gravis
- Opsoclonus-myoclonus syndrome
- Pemphigoid
- 25 Pemphigus
- pernicious anaemia
- Polymyositis/dermatomyositis
- Post-infective arthritides
- Primary biliary sclerosis
- 30 Psoriasis
- Reactive arthritides
- Reiter's disease
- Retinopathy
- Rheumatoid arthritis

- Sclerosing cholangitis
- Sjögren's syndrome
- Stiff-man syndrome
- Subacute thyroiditis
- 5 Systemic lupus erythematosis
- Systemic necrotizing vasculitides
- Systemic sclerosis (scleroderma)
- Takayasu's arteritis
- Temporal arteritis
- 10 Thromboangiitis obliterans
- Type I and type II autoimmune polyglandular syndrome
- Ulcerative colitis
- Uveitis
- Wegener's granulomatosis
- 15 As used herein, the term "ester" refers to a group of the form $-COOR$, wherein R is a radical notionally derived from the alcohol ROH. Examples of ester groups include the physiologically hydrolysable esters such as the methyl, ethyl, n- and iso-propyl, n-, sec- and tert-butyl, and benzyl esters.
- 20 As used herein the term "alkylene" refers to a straight or branched alkyl chain having two unsatisfied valencies, for example $-CH_2-$, $-CH_2CH_2-$, $-CH_2CH_2CH_2-$, $-CH(CH_3)CH_2-$, $-CH(CH_2CH_3)CH_2CH_2CH_2-$, and $-C(CH_3)_3$.
- 25 As used herein the term "alkenylene" refers to a straight or branched alkenyl chain having two unsatisfied valencies, for example $-CH=CH-$, $-CH_2CH=CH-$, $-C(CH_3)=CH-$, and $-CH(CH_2CH_3)CH=CHCH_2-$.
- 30 As used herein the term "alkynylene" refers to a straight or branched alkynyl chain having two unsatisfied valencies, for example $-C\equiv C-$, $-CH_2C\equiv C-$, and $-CH(CH_2CH_3)C\equiv CCH_2-$.

Unless otherwise specified in the context in which it occurs, the term "substituted" as applied to any moiety herein means substituted with at least one substituent, for example selected from (C₁-C₆)alkyl, trifluoromethyl, (C₁-C₆)alkoxy (including the special case where a ring is substituted on adjacent ring C atoms by alkylatedioxy such as methylenedioxy or ethylenedioxy), trifluoromethoxy, (C₁-C₆)alkylthio, phenyl, benzyl, phenoxy, benzyloxy, hydroxy, mercapto, amino, fluoro, chloro, bromo, cyano, nitro, oxo, -COOH, -SO₂OH, -CONH₂, -SO₂NH₂, -COR^A, -COOR^A, -SO₂OR^A, -NHCOR^A, -NHSO₂R^A, -CONHR^A, -SO₂NHR^A, -NHR^A, -NR^AR^B, -CONR^AR^B or -SO₂NR^AR^B wherein R^A and R^B are independently a (C₁-C₆)alkyl or C₂ - C₆ alkoxy group or a monocyclic carbocyclic or heterocyclic group of from 5-7 ring members, or R^A and R^B form a ring when taken together with the nitrogen to which they are attached. In the case where "substituted" means substituted by phenyl, benzyl, phenoxy, or benzyloxy, the phenyl ring thereof may itself be substituted with any of the foregoing, except phenyl, benzyl, phenoxy, or benzyloxy.

As used herein the term "aryl" refers to a mono-, bi- or tri-cyclic carbocyclic aromatic radical, and to two such radicals covalently linked to each other, 20 Illustrative of such radicals are phenyl, biphenyl and napthyl.

As used herein the unqualified term "carbocycll" or "carbocyclic" includes aryl, cycloalkyl and cycloalkenyl and refers to a ring system (monocyclic, 25 bicyclic, tricyclic or bridged) whose ring atoms are all carbon.

As used herein the unqualified term "cycloalkyl" refers to a carbocyclic ring system which contains only single bonds between ring carbons.

As used herein the unqualified term "cycloalkenyl" refers to a carbocyclic ring 30 system which contains at least one double bond between a pair of ring carbons.

As used herein the term "heteroaryl" refers to a mono-, bi- or tri-cyclic aromatic radical containing one or more heteroatoms selected from S, N and

O. Illustrative of such radicals are thienyl, benzthienyl, furyl, benzfuryl, pyrrolyl, imidazolyl, benzimidazolyl, thiazolyl, benzthiazolyl, isothiazolyl, benzisothiazolyl, pyrazolyl, oxazolyl, benzoxazolyl, isoxazolyl, benzisoxazolyl, isothiazolyl, triazolyl, benztriazolyl, thiadiazolyl, oxadiazolyl, pyridinyl, 5 pyridazinyl, pyrimidinyl, pyrazinyl, triazinyl, indolyl and indazolyl.

As used herein the unqualified term "heterocyclyl" or "heterocyclic" includes "heteroaryl" as defined above, and in particular means a mono-, bi- or tricyclic or bridged non-aromatic radical containing one or more heteroatoms

10 selected from S, N and O, and to groups consisting of a monocyclic non-aromatic radical containing one or more such heteroatoms which is covalently linked to another such radical or to a monocyclic carbocyclic radical.

Illustrative of such radicals are pyrrolyl, furanyl, thienyl, piperidinyl, imidazolyl, oxazolyl, isoxazolyl, thiazolyl, thiadiazolyl, pyrazolyl, pyridinyl, pyrrolidinyl, 15 pyrimidinyl, morpholinyl, piperazinyl, indolyl, morpholinyl, benzfuranyl, pyranyl, isoxazolyl, benzimidazolyl, methylenedioxyphenyl, ethylenedioxyphenyl, maleimido and succinimido groups.

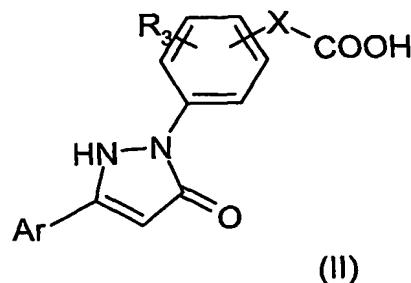
Some compounds of the invention contain one or more chiral centres because 20 of the presence of asymmetric carbon atoms. The presence of asymmetric carbon atoms gives rise to stereoisomers or diastereoisomers with R or S stereochemistry at each chiral centre. The invention includes all such stereoisomers and diastereoisomers and mixtures thereof.

25 Salts of salt forming compounds of the invention include physiologically acceptable acid addition salts for example hydrochlorides, hydrobromides, sulphates, methane sulphonates, p-toluenesulphonates, phosphates, acetates, citrates, succinates, lactates, tartrates, fumarates and maleates; and base addition salts, for example sodium, potassium, magnesium, and calcium 30 salts.

Methods

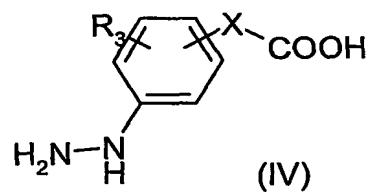
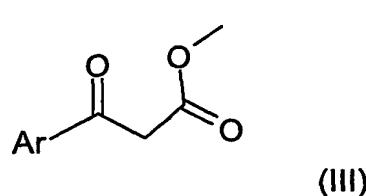
Compounds of the invention of formula (IA) wherein R₁ is hydrogen and wherein R₄ represents an amide group

$-\text{C}(=\text{O})\text{NR}_6\text{R}_7$ may be prepared by reaction of the appropriate amine HNR_6R_7 with a compound of formula (II) to amide the carboxylic acid group:

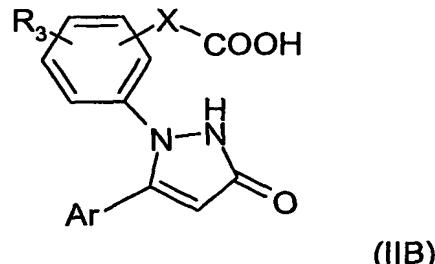
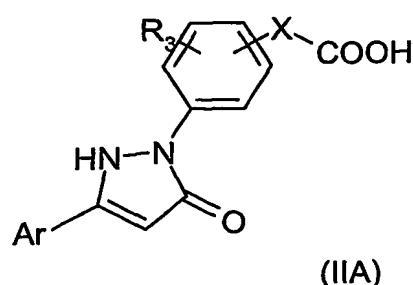


5 the symbols Ar, R_3 , X, R_6 and R_7 being as defined in relation to formula (I) above.

Compounds (II) (ie compounds (IA) of the invention wherein R_1 is hydrogen and R_4 is a carboxylic acid group) may be prepared by reaction of a
10 compound of formula (III) with a hydrazine of formula (IV):

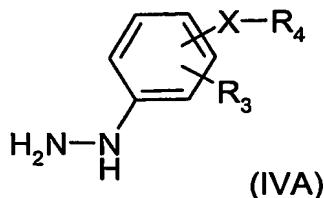


This reaction may result in the preparation of a mixture of the position isomers (IIA) and (IIB):



15 from which the desired isomer (IIA) may be separated.

Compounds (IA) wherein R₁ is hydrogen and R₄ is an amide (−C(=O)NR₆R₇) or ester group may also be prepared from intermediate (III) by reaction with the appropriate hydrazine (IVA)

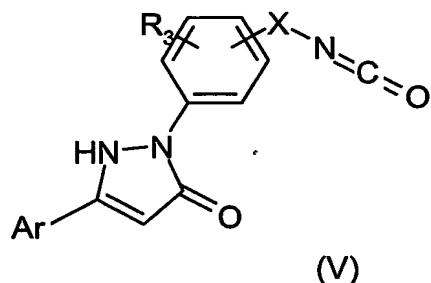


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wherein R₄ is the amide or ester group. Again the reaction may result in a mixture of the ester analogues of the carboxylic acids (IIA) and (IIB), from which the desired ester isomer (I) may be separated. Alternatively, the carboxylic acid compound (II) may simply be esterified, or amidated (the latter being a route referred to above).

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Compounds (IA) wherein R₁ is hydrogen and R₄ is a “reverse amide” group – NR₇C(=O)R₆ may be prepared by Curtius rearrangement (see Ninomiya, K.; Shioiri, T.; Yamada, S. *Tetrahedron* (1974), 30(14), 2151-7) of the carboxylic acid (II) to the isocyanate (V)



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followed by hydrolysis of the isocyanate group to an amino group and acylation of the amino group with, for example, the acid chloride Cl-C(=O)R₆. In cases where R₇ is not hydrogen, the R₇ substituent may be introduced after the isocyanate reduction step or after the acylation step.

Compounds (IA) wherein R₁ is hydrogen and R₄ is a urea group – NHC(=O)NHR₆ or thiourea group –NHC(=S)NHR₆ may also be prepared from

the isocyanate (V) or the corresponding isothiocyanate by reaction with the appropriate amine H_2NR_6 .

5 Compounds (I) wherein R_4 is a carbamate group $-NR_7C(=O)OR_6$ may be prepared by the reaction of the isocyanate with an appropriate alcohol R_6OH .

Compounds (IA) and (IB) wherein R_1 and R_2 are C_1-C_6 alkyl may be prepared by alkylation of the corresponding compound (IA) wherein R_1 is hydrogen.

10 Further details of the synthetic methods for the preparation of compounds (I) of the invention, and intermediates such as (III), may be found in the examples herein.

In the compounds of the invention:

15 R_1 and R_2 independently represent hydrogen or C_1-C_6 alkyl, such as methyl, ethyl, n- or iso-propyl, n-, sec- or tert-butyl.

20 R_4 represents a carboxylic acid group ($-COOH$) or an ester thereof, or $-C(=O)NR_6R_7$, $-NR_7C(=O)R_6$, $-NR_7C(=O)OR_6$ or $-NHC(=O)NHR_6$, all as defined above.

25 When R_4 is an ester group, examples include those of formula $-COOR$ wherein R is methyl, ethyl n- or iso-propyl, n-, sec- or tert-butyl, or benzyl ester.

30 R_6 , when present, represents H, or a radical of formula $-(Alk)_m-Q$ wherein m , Alk and Q being as defined above. When m is 1, Alk may be, for example a straight or branched C_1-C_6 alkylene radical, such as $-CH_2-$, $-CH_2CH_2-$, $-CH_2CH_2CH_2-$, and $-CH_2CH(CH_3)CH_2-$. Alk may also be, for example, a divalent cyclopropylene, cyclopentylene or cyclohexylene radical. The radical Alk may be optionally substituted by, for example, OH, oxo, CF_3 , methoxy or ethoxy. The radical Alk may

optionally contain a hetero atom, for example in the form of an ether, thioether or amino linkage.

5 The group Q may represent, for example, hydrogen; $-NR_8R_8$ wherein each R_8 may be the same or different and selected from hydrogen, methyl, ethyl, n- or isopropyl or tert-butyl; an ester group for example a methyl, ethyl or benzyl ester; or an optionally substituted aryl, aryloxy, cycloalkyl, cycloalkenyl or heterocyclic group, for example phenyl, phenoxy, cyclopentyl, cyclohexyl, furyl, thienyl, piperidyl, or piperazinyl 10 group.

15 R_7 when present represents H or C_1-C_6 alkyl, for example methyl, ethyl n- or iso-propyl, n-, sec- or tert-butyl; or when taken together with the atom or atoms to which they are attached R_6 and R_7 form a monocyclic heterocyclic ring having 5, 6 or 7 ring atoms;

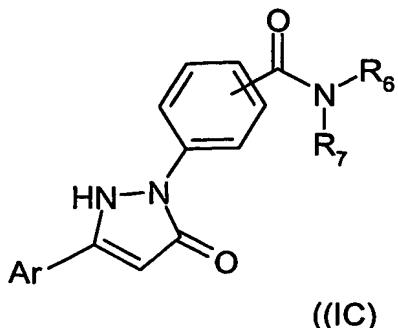
20 Ar may be, for example, optionally substituted phenyl, 2-, 3-, or 4-pyridyl, 2-, or 3-furyl, 2-, or 3-thienyl, benzfur-2-yl, or benzthien-2-yl. Optional substituents in Ar include, for example F, Cl, methyl, methoxy, or methylenedioxy.

Currently it is preferred that Ar is 3-fluorophenyl and 2-, or 3-furyl,

25 R_3 may be, for example, H, F, Cl, methyl, methoxy, or methylenedioxy. Currently it is preferred that R_3 is H;

X may be, for example a bond, or a $-CH_2-$ or $-CH_2CH_2-$ radical. A bond is presently preferred.

A specific preferred subset of compounds of the invention has formula (IC):



wherein Ar, R₆ and R₇ are as specified above. In this subset, the radical –C(=O)NR₆R₇ may be in the 4-position of the phenyl ring. This subset includes in particular, compounds wherein R₇ is hydrogen and R₆ is -AlkNR₈R₈ wherein the R₈ groups are as defined above.

Specific compounds of the invention include those of the Examples herein.

As mentioned above, the invention includes pharmaceutical or veterinary

10 composition comprising a compound of formula (I) or a pharmaceutically or
veterinarily acceptable salt thereof together with a pharmaceutically or
veterinarily acceptable excipient or carrier. In such compositions, it will be
understood that the specific dose level for any particular patient will depend
upon a variety of factors including the activity of the specific compound

15 employed, the age, body weight, general health, sex, diet, time of
administration, route of administration, rate of excretion, drug combination and
the cause and severity of the particular disease undergoing therapy. Optimum
dose levels and frequency of dosing will be determined by clinical trial.

20 The compounds with which the invention is concerned may be prepared for
administration by any route consistent with their pharmacokinetic properties.
The orally administrable compositions may be in the form of tablets, capsules,
powders, granules, lozenges, liquid or gel preparations, such as oral, topical,
or sterile parenteral solutions or suspensions. Tablets and capsules for oral
25 administration may be in unit dose presentation form, and may contain
conventional excipients such as binding agents, for example syrup, acacia,
gelatin, sorbitol, tragacanth, or polyvinyl-pyrrolidone; fillers for example

lactose, sugar, maize-starch, calcium phosphate, sorbitol or glycine; tabletting lubricant, for example magnesium stearate, talc, polyethylene glycol or silica; disintegrants for example potato starch, or acceptable wetting agents such as sodium lauryl sulphate. The tablets may be coated according to methods well

5 known in normal pharmaceutical practice. Oral liquid preparations may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups or elixirs, or may be presented as a dry product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may contain conventional additives such as suspending agents, for example

10 sorbitol, syrup, methyl cellulose, glucose syrup, gelatin hydrogenated edible fats; emulsifying agents, for example lecithin, sorbitan monooleate, or acacia; non-aqueous vehicles (which may include edible oils), for example almond oil, fractionated coconut oil, oily esters such as glycerine, propylene glycol, or ethyl alcohol; preservatives, for example methyl or propyl p-hydroxybenzoate

15 or sorbic acid, and if desired conventional flavouring or colouring agents.

For topical application to the skin, the drug may be made up into a cream, lotion or ointment. Cream or ointment formulations which may be used for the drug are conventional formulations well known in the art, for example as

20 described in standard textbooks of pharmaceutics such as the British Pharmacopoeia.

For topical application to the eye, the drug may be made up into a solution or suspension in a suitable sterile aqueous or non aqueous vehicle. Additives,

25 for instance buffers such as sodium metabisulphite or disodium edetate; preservatives including bactericidal and fungicidal agents such as phenyl mercuric acetate or nitrate, benzalkonium chloride or chlorhexidine, and thickening agents such as hypromellose may also be included.

30 The active ingredient may also be administered parenterally in a sterile medium. Depending on the vehicle and concentration used, the drug can either be suspended or dissolved in the vehicle. Advantageously, adjuvants such as a local anaesthetic, preservative and buffering agents can be dissolved in the vehicle.

Embodiments of the invention are described in the following non-limiting

Examples:

The following abbreviations are used in the experimental descriptions:

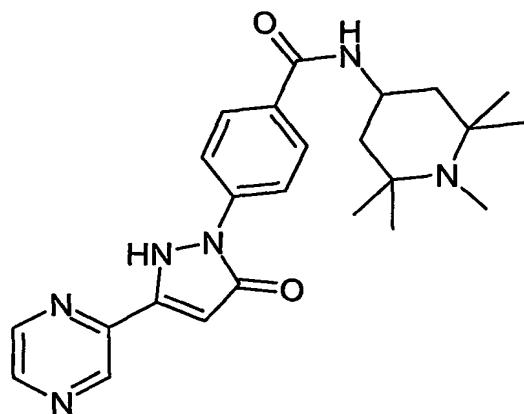
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|------|---|
| DMF | Dimethyl formamide |
| DMA | Dimethyl acetamide |
| DMSO | Dimethyl sulphoxide |
| THF | Tetrahydrofuran |
| HBTU | O-Benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate |
| HPLC | High performance liquid chromatography |
| LCMS | Liquid chromatography mass spectrum |
| NMR | Nuclear magnetic resonance spectroscopy |

Example 1

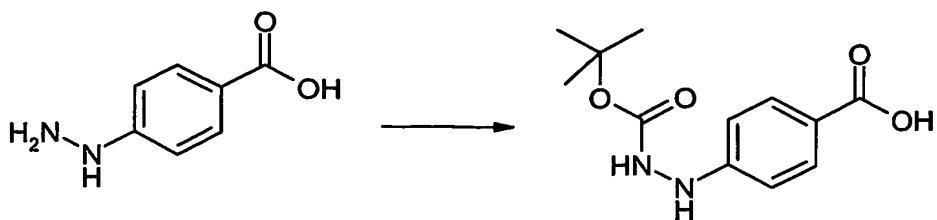
4-(5-Oxo-3-pyrazin-2-yl-2,5-dihydro-pyrazol-1-yl)-N-(1,2,2,6,6-pentamethyl-piperidin-4-yl)-benzamide

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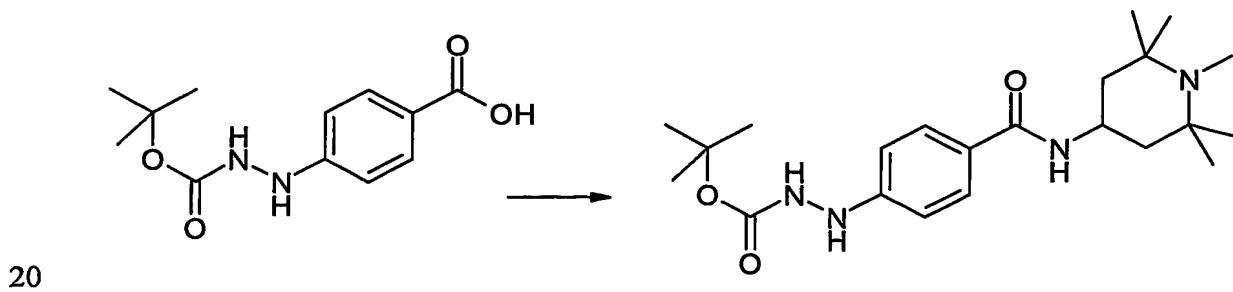
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Step 1: Preparation of 4-(N'-tert-Butoxycarbonyl-hydrazino)-benzoic acid



To a deep red solution of p-hydrazinobenzoic acid (32.86 mmol, 5.00 g) in 2M 5 NaOH (100 ml) and THF (70 ml) was added di-tertbutyl dicarbonate (2.0 eq, 65.72 mmol, 14.34 g). This was stirred at rt over a weekend. Another aliquot of di-tertbutyl dicarbonate (3.5 g) was added to the reaction mixture, and this stirred at room temp for a further 24 hours. A further 0.5 eq p- 10 hydrazinobenzoic acid added to the reaction mixture. This was then stirred for a further 24 hours. 10% citric acid solution was added until a pH of 4 was obtained. The product was then extracted with ethyl acetate, the organics 15 washed with brine, and then dried over Na_2SO_4 . Concentration *in vacuo* afforded a pale yellow oil, containing residual tert-BuOH. Hexane was added to the mixture. Precipitation was observed and the solids were collected by filtration, washed with hexane and dried under vacuum at 35°C. An orange powder was obtained (8.40 g).

Step 2: Preparation of N'-[4-(1,2,2,6,6-Pentamethyl-piperidin-4-ylcarbamoyl)-phenyl]-hydrazinecarboxylic acid tert-butyl ester

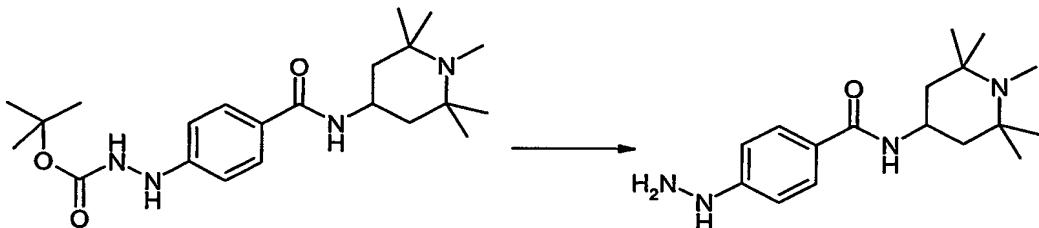


To a solution of 4-(N'-tert-Butoxycarbonyl-hydrazino)-benzoic acid (4.452g) in DMA (30ml) was added disopropyl ethyl amine (6.16ml) and HBTU (6.69g) with stirring at room temp. 4-Amino-1,2,2,6,6-pentamethylpiperidine (3g) was 25 then added to the stirred solution. Stirring continued at room temp for 4hrs.

The solution was partitioned between ethyl acetate (6 X 20 ml) and water. The organic fraction was taken, washed with brine, dried over MgSO_4 and the solvent removed under vacuum. This revealed a deep orange oil (6.2g, 86%).

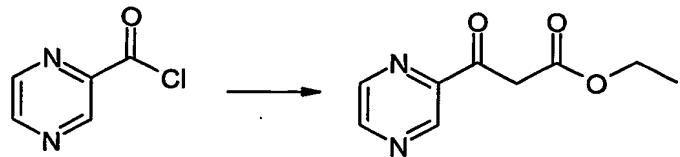
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Step 3: Preparation of 4-Hydrazino-N-(1,2,2,6,6-pentamethyl-piperidin-4-yl)-benzamide



10 $\text{N}'\text{-[4-(1,2,2,6,6-Pentamethyl-piperidin-4-ylcarbamoyl)-phenyl]-hydrazinecarboxylic acid tert-butyl ester}$ was dissolved in 4M HCl in dioxane (30ml) and left to stir for 1-2hrs. The solution went quickly from a deep orange colour to a bright orange colour. After 1hr reaction time, the solvent was removed under vacuum to reveal an orange oil that under a vacuum for 15 several minutes crystallised out to give orange crystals in a sticky orange oil. Yield: 4.68 g, 87%.

20 **Step 4: Preparation of 3-Oxo-3-pyrazin-2-yl-propionic acid, ethyl ester.**



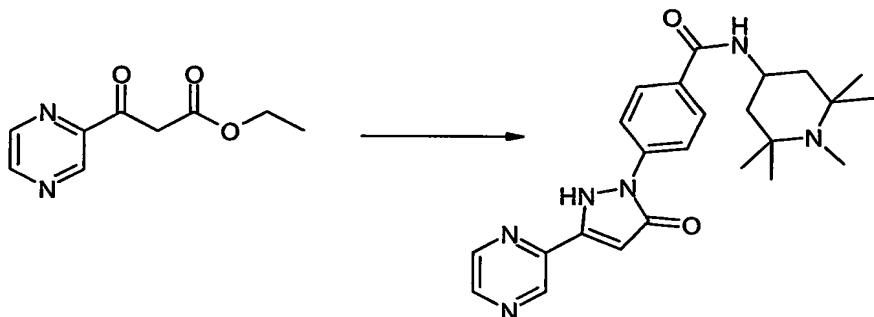
25 To a stirred solution of diethyl malonate (1.25 ml, 1.32g) in THF (20 ml) under an atmosphere of nitrogen at -60°C was added nbutyl lithium (2.5 M, 9 ml) dropwise over 15 min, keeping the temperature constant. The reaction

became a cloudy white colour from the formation of the di-lithium salt then became yellowish. After 10 min, the reaction was cooled to -78°C, when a solution of pyrazine-2-carbonyl chloride (0.5 g, dark purple solution in THF (10 ml)) was added dropwise over 15 min. The reaction then warmed to -45°C

5 and stirred for 1 h. The reaction was then poured into 1M HCl solution (35 ml) with stirring. This was transferred to a separating funnel, and extracted with CH₂Cl₂ (2 x 150 ml). The combined organic layers were then washed with sat. NaCO₃ soln (1 x 30 ml) and then dried over MgSO₄, filtered and concentrated *in vacuo* to give the product as a brown oil. Yield 0.65g, 45%.

10

Step 5: Preparation of 4-(5-Oxo-3-pyrazin-2-yl-2,5-dihydro-pyrazol-1-yl)-N-(1,2,2,6,6-pentamethyl-piperidin-4-yl)-benzamide



15

3-Oxo-3-pyrazin-2-yl-propionic acid, ethyl ester (78 mg) and 4-hydrazino-N-(1,2,2,6,6-pentamethyl-piperidin-4-yl)-benzamide (141mg) were dissolved in ethanol (3.5 ml) and acetic acid (0.5 ml) and the solution stirred at 65 °C for 2h. Concentration *in vacuo* and purification by HPLC gave the expected

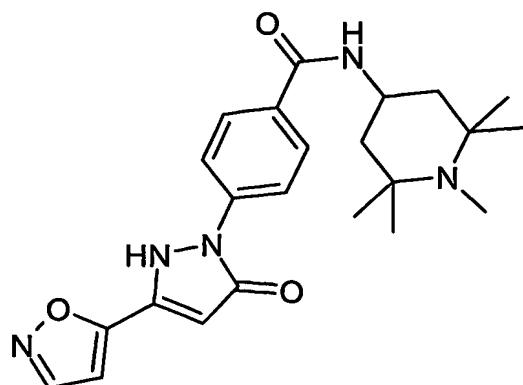
20 product. LC/MS: main peak is product (m/z: 434)

Example 2

4-(3-Isoxazol-5-yl-5-oxo-2,5-dihydro-pyrazol-1-yl)-N-(1,2,2,6,6-pentamethyl-piperidin-4-yl)-benzamide

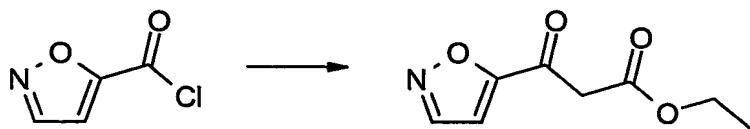
25

19



Step 1: Preparation of 3-Isoxazol-5-yl-3-oxo-propionic acid ethyl ester

5

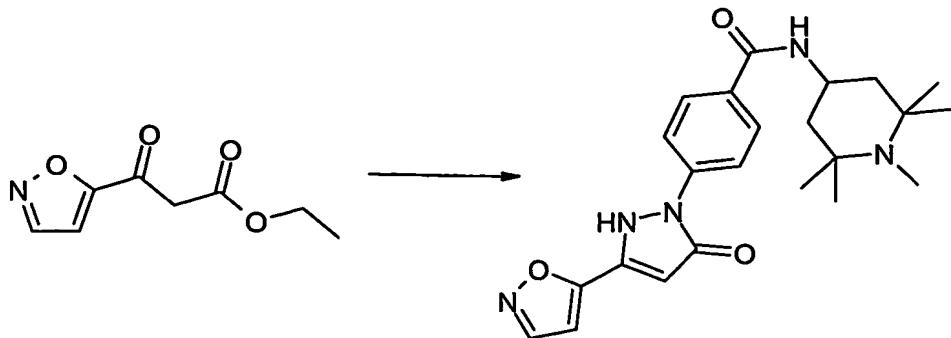


To a stirred solution of diethyl malonate (2.6 ml, 2.74g) in THF (40 ml) under an atmosphere of nitrogen at -60°C was added *n*butyl lithium (2.5 M, 18.9 ml) dropwise over 15 mins, keeping the temperature constant. The reaction

10 became a cloudy white colour from the formation of the di-lithium salt then became yellowish. After 10 mins, the reaction was cooled to -78°C, when a solution of Isoxazole-5-carbonyl chloride (1 g) in THF (10 ml) was added dropwise over 15 mins. The reaction then warmed to -45°C and stirred for 1 h, the solution had gone pale brown. The reaction was then poured into 1M HCl
 15 solution (50 ml) with stirring and extracted with CH₂Cl₂ (2 x 150 ml). The combined organic layers were washed with sat. NaCO₃ soln (1 x 30 ml), dried over MgSO₄, filtered and concentrated *in vacuo* to give the product as a pale oil which was used as such in the next step. Yield 1.1g, 35.1%.

20

Step 2: Preparation of 4-(3-Isoxazol-5-yl-5-oxo-2,5-dihydro-pyrazol-1-yl)-N-(1,2,2,6,6-pentamethyl-piperidin-4-yl)-benzamide



3-Isoxazol-5-yl-3-oxo-propionic acid ethyl ester (49 mg) and 4-Hydrazino-N-(1,2,2,6,6-pentamethyl-piperidin-4-yl)-benzamide (93 mg) were dissolved into ethanol (2.5 ml) and acetic acid (0.3 ml) and the solution stirred at 65 °C for

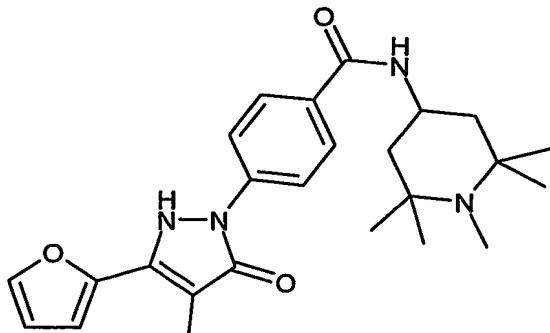
5 2h. Concentration *in vacuo* and purification by HPLC gave the expected product. LC/MS: main peak is product (m/z: 424.3)

The compound of Example 2 had activity rating * in the HTRF assay described below.

10

Example 3

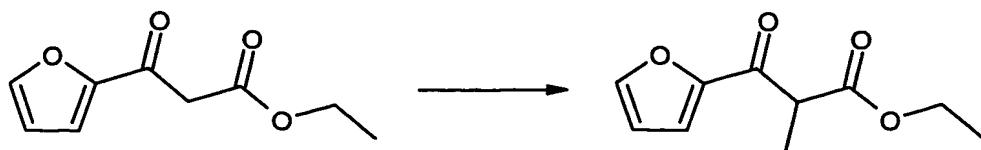
4-(3-Furan-2-yl-4-methyl-5-oxo-2,5-dihydro-pyrazol-1-yl)-N-(1,2,2,6,6-pentamethyl-piperidin-4-yl)-benzamide



15

Step 1: Preparation of 3-furan-2-yl-2-methyl-3-oxo-propionic acid ethyl ester

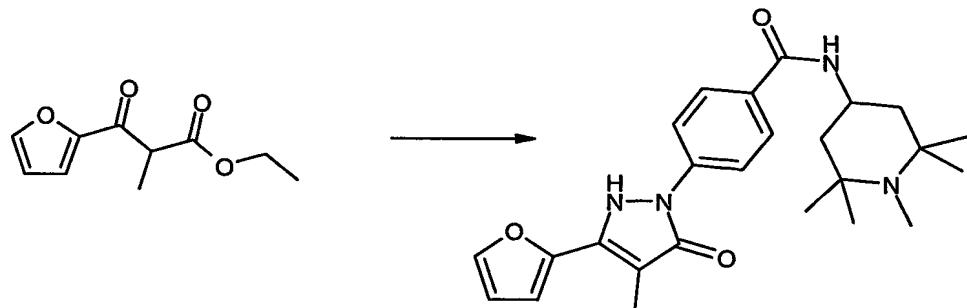
20



In a flask were placed ethyl 3-(2-furyl)-3-oxopropanoate (0.5g), iodomethane (0.14 ml), finely ground potassium carbonate (0.75g) and acetone (5 ml). The mixture was stirred at reflux for 2h. A further portion of iodomethane

5 (0.9mmol, 0.056 ml) and potassium carbonate (0.9 mmol, 0.12g) were added and the mixture stirred at reflux for 1 h. The reaction mixture was filtered and concentrated under vacuum. Yield (0.5g, 93%).

Preparation of 4-(3-Furan-2-yl-4-methyl-5-oxo-2,5-dihydro-pyrazol-1-yl)-N-(1,2,2,6,6-pentamethyl-piperidin-4-yl)-benzamide



10

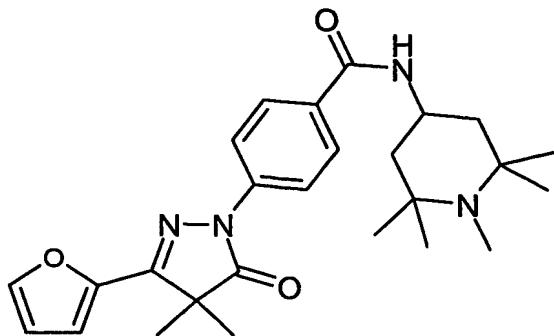
3-furan-2-yl-2-methyl-3-oxo-propionic acid ethyl ester (49 mg) and 4-Hydrazino-N-(1,2,2,6,6-pentamethyl-piperidin-4-yl)-benzamide (85 mg) in 0.5 ml of acetic acid were heated up at 65 °C for 2h. Concentration *in vacuo* and purification by HPLC gave the expected product. $MH^+ = 437.3$

15

The compound of Example 3 had activity rating * in the HTRF assay described below.

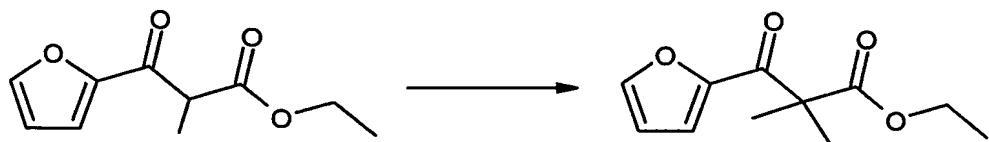
Example 4

20 **4-(3-Furan-2-yl-4,4-dimethyl-5-oxo-4,5-dihydro-pyrazol-1-yl)-N-(1,2,2,6,6-pentamethyl-piperidin-4-yl)-benzamide**



Step 1: Preparation of 3-Furan-2-yl-2,2-dimethyl-3-oxo-propionic acid ethyl ester

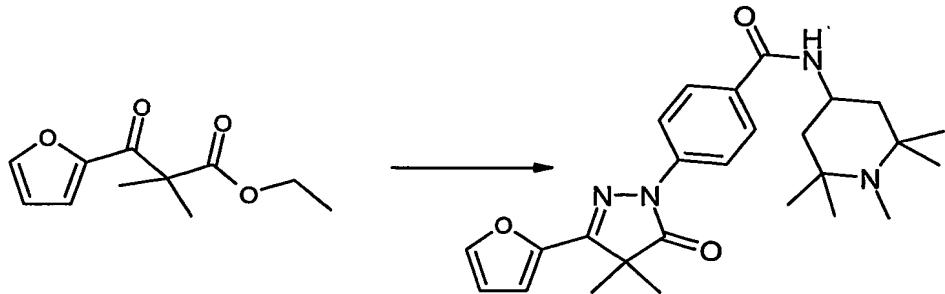
5



3-furan-2-yl-2-methyl-3-oxo-propionic acid ethyl ester (49 mg) was added sodium ethoxide (0.22 ml) and 0.5 ml ethanol. A solid precipitates. The

10 solution was stirred at room temperature for 1h, iodomethane was added and the suspension stirred at room temperature for 3 h. Concentration *in vacuo* and purification by HPLC gave the expected product.

15 **Step 2: Preparation of 4-(3-Furan-2-yl-4,4-dimethyl-5-oxo-4,5-dihydro-pyrazol-1-yl)-N-(1,2,2,6,6-pentamethyl-piperidin-4-yl)-benzamide**



3-Furan-2-yl-2,2-dimethyl-3-oxo-propionic acid ethyl ester (53 mg) and 4-Hydrazino-N-(1,2,2,6,6-pentamethyl-piperidin-4-yl)-benzamide (85 mg) in 0.5 ml of acetic acid were heated up at 65 °C for 2h. Concentration *in vacuo* and purification by HPLC gave the expected product (MH⁺: 451.4)

5

Additional Examples

Further examples of compounds of the invention were synthesised by methods analogous to those of Examples 1 - 2 above. The structures of the synthesised compounds are shown in the following Table, together with their

10 activity ratings in the HTRF assay described below.

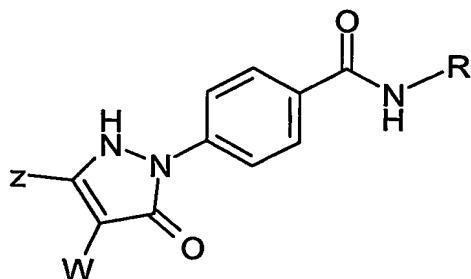
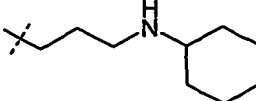
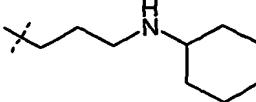
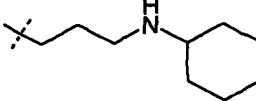
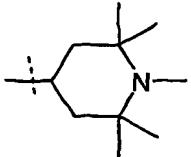
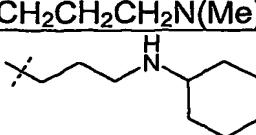
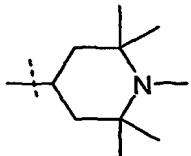
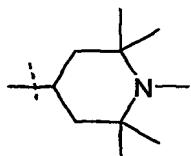
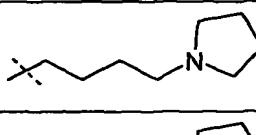
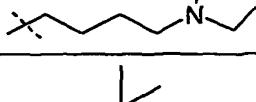
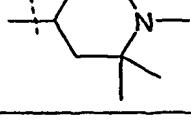
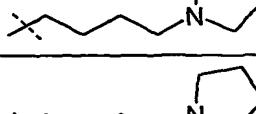
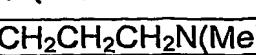
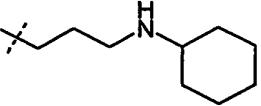
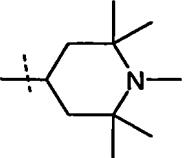
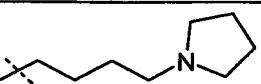
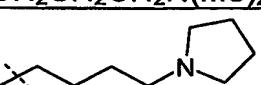
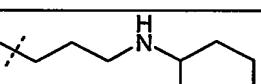
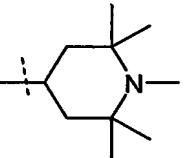
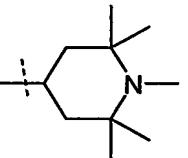


Table 1

| Example | Z | W | R | MH ⁺ | Activity Rating |
|---------|-----------|---|--|-----------------|-----------------|
| 5 | Ph | H | CH ₂ CH ₂ CH ₂ N(Me) ₂ | 365.2 | ** |
| 6 | Ph | H | | 482.1 | * |
| 7 | Ph | H | | 433.5 | ** |
| 8 | Ph | H | | 405.5 | *** |
| 9 | Ph | H | | 419.5 | ** |
| 10 | 2-Pyridyl | H | CH ₂ CH ₂ CH ₂ N(Me) ₂ | 366.5 | *** |
| 11 | 2-furyl | H | CH ₂ CH ₂ CH ₂ N(Me) ₂ | 355.4 | *** |
| 12 | 3-Pyridyl | H | CH ₂ CH ₂ CH ₂ N(Me) ₂ | 366.5 | ** |

| | | | | | |
|----|-----------|---|--|-------|-----|
| 13 | 2-Pyridyl | H |  | 420.5 | ** |
| 14 | 2-furyl | H |  | 409.5 | *** |
| 15 | 3-Pyridyl | H |  | 420.5 | ** |
| 16 | 2-Pyridyl | H |  | 434.6 | *** |
| 17 | 3-furyl | H | <chem>CH2CH2CH2N(Me)2</chem> | 355.5 | ** |
| 18 | 3-furyl | H |  | 409.5 | *** |
| 19 | 2-furyl | H |  | 423.6 | *** |
| 20 | 3-furyl | H |  | 423.6 | ** |
| 21 | 2-furyl | H |  | 395.6 | ** |
| 22 | 3-furyl | H |  | 395.6 | ** |
| 23 | 3-Pyridyl | H |  | 434.6 | ** |
| 24 | 2-Pyridyl | H |  | 406.5 | *** |
| 25 | 3-Pyridyl | H |  | 406.6 | ** |
| 26 | 3F-phenyl | H | <chem>CH2CH2CH2N(Me)2</chem> | 383.5 | *** |

| | | | | | |
|----|----------------|---|---|-------|-----|
| 27 | 3F-phenyl | H |  | 437.6 | ** |
| 28 | 3F-phenyl | H |  | 451.6 | *** |
| 29 | 2-pyrazinyl | H |  | 407.3 | ** |
| 30 | 2-pyrazinyl | H | <chem>CH2CH2CH2N(Me)2</chem> | 367.3 | *** |
| 31 | 2-benzofuranyl | H |  | 445.3 | ** |
| 32 | 2-benzofuranyl | H | <chem>CH2CH2CH2N(Me)2</chem> | 405.3 | ** |
| 33 | 2-benzofuranyl | H |  | 459.4 | ** |
| 34 | 2-benzofuranyl | H |  | 473.4 | ** |
| 35 | 5-isoxazolyl | H |  | 424.3 | * |

Assay Protocols

5 The use of BIACore biomolecular interaction analysis

Biotinylated human CD80 (hCD80-BT) is a recombinant soluble form of a membrane bound receptor molecule (CD80) which binds to CD28 to initiate T cell activation. The interaction between CD80 and CD28 has been extensively investigated (Collins et al, 2002). Biotinylated human HLA-A2-tax is the

10 recombinant soluble form of a membrane bound receptor molecule that has been used in this example as a control protein, and is not expected to interact with the compounds.

The BIACore S51TM system was used for screening the compounds of Examples 1-4 above. A series S sensor chip CM5 was docked onto the BIACore S51TM. Streptavidin was coupled to the carboxymethyl surface using standard amine coupling. The chip surface was activated with 0.2M EDC / 0.05M NHS, followed by binding of streptavidin (0.25 mg/ml in 10 mM sodium acetate pH 5.0) and saturation of unoccupied sites with 1 M ethylenediamine.

5 The BIACore S51 sensor chip has two separate sensor spots for immobilisation of proteins. hCD80-BT was immobilised on the streptavidin-coated surface of one sensor spot until a response of approximately 3000 RU was observed. A protein to control for non-specific binding of the compound was immobilised on a second sensor spot. The control protein used for these experiments was a biotinylated, soluble form of the human HLA protein.

10 15 Dilution series of compounds (1000nM – 0.05nM) were prepared in running buffer (10 mM, pH 7.4, 150 mM NaCl, 0.005% P20; 5% DMSO).

20 BIACore S51TM was run at a flow rate of 30 µl/min using running buffer. Compounds and DMSO standard solutions for correction of data for solvent effects were injected. Data were recorded automatically and were analysed using BIACore S51 Evaluation software.

25 The interaction between CD80 and the endogenous protein ligand (CD28) is highly specific, but relatively weak, with a K_D of 4750 nM, and an off-rate of greater than 0.2 s⁻¹. The compounds of Examples 7, 11 & 18-21 have greater affinity and longer residence times on CD80 than CD28, having K_{D5} of less than 100nM, and off-rates of 2x10⁻², indicating that the pyrazolones will be able to compete effectively with the endogenous ligand. The pyrazolones 30 showed no detectable interaction with the control protein.

References

Collins AV *et al.* (2002) *Immunity* 17, 201-210 "The interaction properties of costimulatory molecules revisited"

Inhibition of production of interleukin-2 (IL-2) by human Jurkat T cells.

5 **Method**

Human Raji cells were dispensed at a concentration of 2×10^5 cells per well in RPMI-1640 medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, 1% glutamine (RPMI medium) in a 96-well round bottom microtitre plate. Compounds under investigation (dissolved in 100% 10 DMSO) were diluted to eight-fold the desired final concentration in RPMI medium and added to the required final concentration for a total volume of 200 μ l per well. After 20 minutes incubation at 37°C, Jurkat T cells were added at a concentration of 2×10^5 cells per well. Monoclonal antibody to CD3 (UCHT1, R&D Systems) was added to the cultures at a final concentration of 15 1 μ g per ml, and where indicated, monoclonal antibody to CD28 (CD28.2, BD-Pharmingen) was also added at a concentration of 2.5 μ g per ml. Cells were cultured at 37°C for 5 hours, after which the plates were centrifuged and the supernatants harvested for IL-2 ELISA assay using the IL-2 Eli-pair kit (DIACLONE Research, Besancon, France) according to the manufacturers 20 instructions.

By way of example, the compound of Example 26 gave 52% inhibition at 30 μ M.

25 **Homogenous Time Resolved Fluorescence Assay**

The examples described above were tested in a cell free Homogenous Time Resolved Fluorescence (HTRF) assay to determine their activity as inhibitors of the CD80-CD28 interaction.

30 In the assay, europium and allophycocyanin (APC) are associated with CD28 and CD80 indirectly (through antibody linkers) to form a complex, which brings the europium and APC into close proximity to generate a signal. The complex comprises the following six proteins: fluorescent label 1, linker antibody 1,

CD28 fusion protein, CD80 fusion protein, linker antibody 2, and fluorescent label 2. The table below describes these reagents in greater detail.

| | |
|---------------------|--|
| Fluorescent label 1 | Anti-Rabbit IgG labelled with Europium (1 μ g/ml) |
| Linker antibody 1 | Rabbit IgG specific for mouse Fc fragment (3 μ g/ml) |
| CD28 fusion protein | CD28 - mouse Fc fragment fusion protein (0.48 μ g/ml) |
| CD80 fusion protein | CD80 mouse Fab fragment (C215) fusion protein (1.9 μ g/ml) |
| Linker antibody 2 | G α M κ -biotin: biotinylated goat IgG specific for mouse kappa chain (2 μ g/ml) |
| Fluorescent label 2 | SA-APC: streptavidin labelled allophycocyanin (8 μ g/ml) |

5 On formation of the complex, europium and APC are brought into proximity and a signal is generated.

Non-specific interaction was measured by substituting a mouse Fab fragment (C215) for the CD80 mouse Fab fragment fusion protein (1.9 μ g/ml). The 10 assay was carried out in black 384 well plates in a final volume of 30 μ l. Assay buffer: 50mM Tris-HCl, 150mM NaCl pH7.8, containing 0.1% BSA (w/v) added just prior to use.

Compounds were added to the above reagents in a concentration series 15 ranging between 100 μ M – 1.7nM. The reaction was incubated for 4 hours at room temperature. Dual measurements were made using a Wallac Victor 1420 Multilabel Counter. First measurement: excitation 340nm, emission 665nm, delay 50 μ s, window time 200 μ s. second measurement: excitation 340nm, emission 615nm, delay 50 μ s, window time 200 μ s. Counts were 20 automatically corrected for fluorescence crossover, quenching and background. The EC50 activities of compounds tested are recorded as: EC50: * = >10 μ M, ** = 1-10 μ M, *** = <1 μ M.